

1    **Repeated electrical stimulations as a tool to evoke temporal**  
2    **summation of nociceptive inputs in healthy, non-medicated**  
3    **experimental sheep**

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21    Running title: repeated electrical stimulations can evoke temporal summation in sheep

## 1. Introduction

The sheep is widely used as an animal model in biomedical research. Experimental trials are often invasive and can only be ethically justified with a continuous refinement of peri-operative pain management according to the 3R principles. Surgical interventions can lead to peripheral and central sensitization, resulting in allodynia and hyperalgesia as a consequence of tissue damage, nerve trauma or inflammation [1, 2].

Some years ago, sheep suffering from foot rot, an infectious pododermatitis, have been used as a model of persistent inflammatory pain [3-6]. Various analgesics as opioids, alpha-2 agonists [4] and non-steroidal anti-inflammatory drugs [3] have been assessed for their efficacy in modifying the behavioural reaction following a painful stimulation of distal affected limbs. Interestingly, nociceptive thresholds remained lower than in healthy animals even after successful treatment of the inflammatory process. In addition, surgically induced peroneal nerve injury leading to neuropathic pain and thus to a significant persistent increase in skin sensitivity has been promoted as a useful model for evaluating analgesics in sheep [7]. Unfortunately, both models are invasive and non-reversible and therefore not appropriate to be applied in refinement studies aiming at improving analgesia in sheep undergoing experimental orthopaedic surgery as animal models.

Until now, a non-invasive model that can be used to evaluate central sensitization as a consequence of orthopaedic surgery or the efficacy of loco-regional and systemic antinociceptive treatments is missing in sheep. The nociceptive withdrawal reflex (NWR) model has been used as a non-invasive neurophysiological tool to assess spinal nociceptive processing in humans and animals [8-12]. In humans, the NWR threshold was found to correlate with the pain threshold [13, 14]. The species-specific characteristics of the NWR after single stimulation have been evaluated in sheep in a previous study [15].

Stimuli of intensity below NWR threshold, insufficient to provoke any response when presented as a single stimulus, were found to summate and lead to increased nociceptive

reflexes and aversive behavioural reactions when administered repeatedly [16-18]. This facilitation of the nociceptive reflex response, originating from the temporal summation of sensory inputs, has been proposed as a non-invasive measure to quantify the physiology of central integrative and sensitization processes associated with the wind-up phenomenon observed in dorsal horn cells to repetitive nociceptive input [19].

According to our hypothesis, sub-threshold repeated electrical stimulations of the lateral digital nerves of the fore limb and the hind limb will summate facilitating the NWR and it will be possible to quantify this facilitation analyzing specific post-stimulation epochs of the electromyographic signals elicited.

Aim of this prospective study was to first define the individual NWR threshold ( $I_t$ ) to single stimulation and then to apply series of 10 repeated stimuli at fractions of  $0.5-1 \times I_t$  to evaluate the extent of temporal summation of nociceptive inputs in conscious, non-medicated standing sheep. Following each stimulation, the evoked behavioural response was observed and scored to be able to complement the information gained from the neurophysiological data.

## 2. Methods

### 2.1. *Animals*

Twenty-five adult Swiss Alpine sheep were included in the study (mean weight: 63.1 kg  $\pm$  6.1 kg (SD); age 2-3 years). All sheep were part of an orthopaedic study starting after this experiment. Prior to the experiment, the animals were clinically examined and kept under close clinical surveillance during the entire experimental period. The experiment was approved by the Committee for Animal Experimentation of the Canton Graubunden, Switzerland. The sheep were housed together in one stable within individual boxes. The room temperature was 16°C. A maintenance diet consisting of a mixture of straw, silage, maize and salt was fed twice daily while water was available ad libitum in an automatic water drinker. Food but not water was withheld in the morning of the experimental session. All measurements were performed during the morning and took place in the stable where the animals had been housed for 4 to 6 weeks. The day before the experiment the sheep were placed into purpose-made suspension slings to guarantee restrain and avoid recumbency during the experiment. Normal standing and limited walking were always possible while being placed in the slings.

### 2.2. *Electrical stimulation and electromyography*

Each experimental session was started at the thoracic limb (deltoid muscle in 25 animals). First, the nociceptive threshold ( $I_t$ ) was determined as previously reported [15]. In brief, nociceptive withdrawal reflexes were evoked in sequence by electrical stimulation of the lateral digital nerves of the thoracic and the pelvic limb. Electromyographic recordings from the deltoid muscle of the thoracic limb and from the biceps femoris and the peroneus tertius of the pelvic limb were used to objectively quantify the reflexes. The behavioural reaction following each stimulation was observed and scored by the same investigator (HR) on a scale from 0 (no reaction) to 5 (strong whole body reaction; Table 1). Threshold intensity ( $I_t$ ) was defined as the

minimal stimulus intensity able to evoke an EMG response of 20  $\mu$ V (RMS<sub>A</sub>) with a duration of at least 10 ms within the NWR interval and a minimal reaction score of 1/5 (Table 1)[14].

In a next step, 10 pulse trains were delivered over a 2s period with a stimulation frequency of 5 Hz. The stimulation intensities applied were fractions of the individual  $I_t$  (0.5, 0.6, 0.7, 0.8, 0.9 and 1 x  $I_t$ ). If a reflex could be detected after stimulation at 0.5 x  $I_t$  evoked by any of the 10 stimuli in the train (EMG, behaviour), the next stimuli were reduced to 0.4 x  $I_t$  and then to 0.3 x  $I_t$  until no reaction or EMG could be evoked anymore. The full stimulus-response curves were acquired to each sheep so all remaining intensities were subsequently applied. Between two stimulation series, intervals of at least 60s were maintained.

After defining the thoracic limb  $I_t$  and the application of the repeated stimulations at the corresponding intensities, the procedure was repeated at the pelvic limb.

### 2.3. Reflex analysis

The pre-stimulation (0-500 ms), the stimulation (500-2500 ms) and the post-stimulation (2500-4000 ms) periods were analysed separately (sampling frequency: 1024 Hz). For the reflex analysis, the stimulation period was divided into the NWR interval (20-130 ms) and the post-NWR interval (130-200 ms) following each pulse train and the nociceptive threshold for repeated stimulations (RS  $I_t$ ) was determined.

The RS  $I_t$  was defined as the minimal stimulus intensity able to evoke one EMG response of a minimal RMS amplitude (RMS<sub>A</sub>) of 20  $\mu$ V with a duration of at least 10 ms within the NWR interval after any of the 10 stimuli and a reaction score of at least 1/5 (Table 1). To better understand the reflex patterns, the amplitude of the EMG activity recorded in the NWR interval after the first stimulus was compared with the amplitude of the maximal NWR within the stimulation series. The post-NWR interval was evaluated to prove the correctness of the NWR interval as only the time window of 20-130 ms was expected to be originated by reflexive activation of A-delta fibres while the window of 130-200 ms could potentially be influenced by supraspinal activation [15].

117 The pre-stimulation period was analysed to prove balanced weight distribution while the  
118 post-stimulation-period was used to determine continued movements after cessation of  
119 stimulation.

#### 121 2.4. Statistical analysis

122 Data analysis was performed using statistical software (Sigma Stat, Version 3.5, Systat  
123 Software). Nonparametric analysis of data was chosen on the basis of tests for normality of  
124 distribution. Friedman repeated measures ANOVA on ranks followed by Tukey test were  
125 applied to compare the effects of stimulation intensity on the reflex characteristics (NWR  
126 interval, post-NWR interval, pre- and post-stimulation periods and behaviour) if significant  
127 overall effects were detected. The Brunner-Langer LD-F2 model [20] was applied to analyse the  
128 effects of repetition of the stimuli, the effects of stimulation intensity on reflex amplitude (NWR  
129 interval) and the interaction between the two variables at the deltoid and the biceps femoris  
130 muscles. Stimulation intensities starting from  $0.5 \times I_t$  were considered for all analyses. Only for  
131 the determination of RS  $I_t$  stimulation intensities at lower fractions were included. The  
132 Wilcoxon Signed Rank Test was used to compare the  $RMS_A$  of the EMG activity following the  
133 first stimulus with the  $RMS_A$  of the maximal NWR within the stimulation series. Significance  
134 was set at  $p < 0.05$ .

### 3. Results

Clinical examination revealed no abnormalities in any animal. The sheep tolerated the experiments well as the observed nocifensive reactions stopped immediately after stimulation. With the current intensities used to determine repeated stimulation thresholds in the present study, none of the sheep appeared distressed after termination of the stimulations. Results are reported in Table 2.

Intensities necessary to evoke a NWR varied among individuals and were muscle-specific. Median  $I_t$  was 4.4 mA (IQR 2.9-5.7 mA) with a behaviour score of 1 (1-2) for the deltoid muscle, 7 mA (IQR 4-10 mA) with a behaviour score of 1 (1-2) for the biceps femoris muscle, and 3.4 mA (IQR 3.05-4.35 mA) with a behaviour score of 1 (1-1) for the peroneus tertius muscle.

For the deltoid muscle, the first reflex (RS  $I_t$ ) occurred at  $0.6 \times I_t$  (0.5-0.8) corresponding to 2.3 mA (1.6-3 mA) with a reaction score of 2 (1-2). For the biceps femoris muscle, RS  $I_t$  was  $0.55 \times I_t$  (0.4-0.7) corresponding to 2.9 mA (2.6-4 mA) with a reaction score of 1 (1-2) while for the peroneus tertius muscle RS  $I_t$  was  $0.8 \times I_t$  (0.8-0.95) corresponding to 3 mA (2.8-3.5 mA) with a reaction score of 1 (1-2). The RS  $I_t$  was lower than  $0.4 \times I_t$  in 3 animals for the deltoid muscle and in 4 animals for the biceps femoris muscle but for the peroneus tertius muscle the lowest detected RS  $I_t$  was  $0.6 \times I_t$ .

The mean  $RMS_A$  of the NWR interval (20-130 ms) increased significantly ( $p < 0.001$ ) when the relative stimulus intensity was increased from 0.5 to  $1 \times I_t$  for all 3 muscles. The reaction score increased with increasing stimulation intensities for all muscles ( $p < 0.001$ ).

The  $RMS_A$  of the pre- (0-500 ms) and the post-stimulation periods (2500-400 ms) remained low despite stimulus intensity increased from 0.5 to  $1 \times I_t$  in all three muscles.

When the stimulus intensity increased from 0.5 to  $1 \times I_t$  a significant intensity effect could be detected for the deltoid and the biceps femoris muscles ( $p < 0.001$ ; Fig. 1). The  $RMS_A$  increased and a NWR could be detected in an increasing proportion of animals. The stimulus

number within the stimulation series had neither any influence on the RMS amplitudes nor on the proportion of animals with a nociceptive reflex ( $p=0.45$ ). When both effects were combined, no effect of interaction was found in any muscle ( $p=0.32$ ).

The EMG activity following the first stimulus was significantly smaller than the maximal NWR for the deltoid and the biceps femoris muscle at all stimulus intensities, while for the peroneus tertius the EMG activity following the first stimulus was also the largest NWR at 0.9 and  $1 \times I_t$ . The proportion of animals in which the first stimulus in the train evoked the largest NWR increased with increasing stimulus intensity for the deltoid and the peroneus muscle, but not for the biceps femoris muscle (Table 3).



## 4. Discussion

In the present study, electrical stimuli of intensity below the previously determined nociceptive threshold for a single stimulus led to temporal summation with an increase in nociceptive reflex amplitude and aversive behavioural reactions when presented repeatedly at a frequency of 5 Hz in conscious, non-medicated, standing sheep.

Repeated stimulation thresholds (RS  $I_t$ ) and the effects of stimulus intensity on reflex amplitude were determined. Reflex recording was accompanied by a scoring of the behavioural reaction following each stimulation series. For the determination of the nociceptive threshold the appearance of a clear behavioural reaction was considered fundamental to be sure that the animal perceived the stimulation as unpleasant. In humans it is known that the stimulation intensity evoking a nociceptive withdrawal reflex on the electromyogram correlates with pain perception [13, 14].

With the current intensities used to elicit nociceptive reflexes in the present study, none of the sheep appeared distressed as the behavioural reactions stopped immediately on stimulation cessation. Even when high stimulus intensities were used, the reaction pattern remained localized to the limb under evaluation.

After determining the  $I_t$  after single stimulation, repeated stimuli were applied and the RS  $I_t$  was defined. The stimulation pattern of 10 stimuli at a frequency of 5 Hz as used in this study has been previously applied to horses [16] and humans [18]. The relative RS  $I_t$  was comparable for the deltoid and for the biceps femoris muscle while it was higher for the peroneus tertius muscle. The lack of reflex facilitation in the peroneus tertius muscle at subthreshold levels might be explained by the functional characteristics of the muscle as a stabilizer rather than as a flexor. As a consequence, recording from the peroneus tertius muscle cannot be recommended for future studies using if the target is to assess spinal nociceptive processing.

Standing equally on 4 limbs was a prerequisite for starting the stimulation procedure. A silent background EMG (judged visually) reduced the occurrence of facilitation due to increased

excitability of the motor neuron pool that might take place in case of spontaneous muscle activity prior to stimulation [21]. Data analysis off-line confirmed that the pre-stimulation period was characterised by a low  $RMS_A$ . For the post-stimulation period, the  $RMS_A$  had a tendency to increase only for the highest stimulation intensities ( $0.9, 1 \times I_t$ ) associated with more intense and prolonged behavioural reactions.

In humans, repeated stimulations at intensities below the individual pain threshold were able to elicit gradually larger NWR [18] most likely reflecting temporal summation in deep dorsal horn neurons due to central integration of the neural responses leading to an increasing pain sensation [18, 22]. Temporal summation seems to reflect the early phase of wind-up, a phenomenon observed in rats [19] which is part of the pathophysiologic basis of clinical persistent pain syndromes [23]. In sheep, the number of stimuli had no influence on the mean  $RMS_A$ . Anyway, the amplitude of the maximal reflex was significantly larger than the amplitude of the first reflex which reflects induction of temporal summation as seen in humans. Increasing stimulation intensity towards intensity threshold ( $1 \times I_t$ ) increased the number of animals showing the maximal reflex after the first stimulus as presented in Table 3. For the deltoid and the peroneus tertius muscle this phenomenon was evident while for the biceps femoris muscle a further increase of the reflex amplitude was still observed at the highest stimulation intensities even if the first stimulus was already able to evoke a nociceptive reflex. This muscle specific response is probably linked to the topographical organization of the nociceptive reflex in sheep [24].

The epoch of 20-130 ms post-stimulation used in this study to identify the NWR indicates that most probably the reflex originates from activation of A-delta fibres [15]. Wind-up was originally believed to result from repetitive C-fibre stimulation only [25] but when A-delta fibres were stimulated at a minimal frequency of 0.3 Hz an occurrence of wind-up occurs was demonstrated for these fibres as well [26]. C-fibre stimulation would require stimulation for several seconds at an intensity not tolerated by awake subjects [18]. Therefore, the proposed method can be considered as an adequate non-invasive model of wind-up. The goal of this study

was to generate reference values for later applications of this model in experimental sheep undergoing orthopaedic procedures. In humans, a segmental sensitization could be detected in the immediate post-operative period [27] . When a local anaesthetic was administered to the epidural space, pain sensation to single stimulation was inhibited in 6/10 human volunteers while it was only abolished in 1/10 of the same subjects if repeated electrical stimuli were applied [28]. This discrepancy has been explained with changes in the excitability of neurons on spinal level induced by repeated stimulations and confirms the importance of a multimodal set of stimulation modalities.

## **Conclusions**

The repeated application of stimuli at intensity unable to evoke a nociceptive withdrawal reflex if given singularly, led to increasing amplitudes of the nociceptive reflexes and aversive behavioural reactions and therefore temporal summation in healthy standing sheep without inducing persisting stress and discomfort for the animal. Data achieved in this study can serve as reference values for future experimental applications of the model in this species.

## **Conflict of interest statement**

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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